

Diagnosis of Hydatidiform Moles by Polymorphic Deletion Probe Fluorescence *in Situ* Hybridization

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Because products of conception often contain maternal and villous tissues, the determination of maternal and villous genotypes based on genetic polymorphisms can help discern maternal and paternal chromosomal contribution and aid in the diagnosis of hydatidiform moles. Polymorphic deletion probe (PDP) fluorescence *in situ* hybridization (FISH) probes based on copy number variants are highly polymorphic and allow *in situ* determination of genetic identity. By using three informative PDPs on chromosomes 2p, 4q, and 8p, we compared maternal with villous genotypes and determined the ploidy of villous tissue. PDP FISH was performed on 13 complete moles, 13 partial moles, 13 nonmolar abortions, and an equivocal hydropic abortion. PDP FISH permitted definitive diagnosis of complete moles in five of 13 cases for which maternal and villous genotypes were mutually exclusive. A complete mole was highly suspected when all three PDP loci showed homozygous villous genotypes. The diagnosis of a complete mole by PDP FISH yielded a theoretical test sensitivity of 87.5%, specificity of 91.8%, an observed test sensitivity of 100%, and specificity of 92.3%. Triploidy was observed in all partial moles, in which diandric triploidy was confirmed in six cases. In the equivocal hydropic abortion, PDP FISH combined with p57 immunofluorescence revealed placental androgenetic/biparental mosaicism. PDP FISH can be used in clinical practice and research studies to subclassify hydatidiform moles and evaluate unusual products of conception. (*J Mol Diagn* 2011, 13:406–415; DOI: 10.1016/j.jmoldx.2011.02.002)

Hydatidiform moles (HMs) are a type of gestational trophoblastic disease that results from abnormal fertilization and subsequent trophoblastic proliferation. In the United States, the incidence of HMs is approximately 0.1%, ranging from 108 to 121 per 100,000 pregnancies.^{1–3} By clinicopathologic features and karyotype analysis, HMs can be subclassified into two distinct groups: complete HMs (CHMs) and partial HMs (PHMs). The distinction between CHMs and PHMs is clinically important because of the risks of recurrence, persistent gestational trophoblastic disease, and malignant transformation. Patients with a history of any single molar pregnancy have a 1% risk of recurrence with a subsequent pregnancy; those with a history of two molar pregnancies have a 10% to 28% risk.^{4–8} The incidence of persistent gestational trophoblastic disease after a CHM is reportedly 18% to 29%,^{9–12} and the risk after a PHM is 0% to 11%.^{13,14} The incidence of choriocarcinoma in the United States is 0.18 per 100,000 pregnancies.¹⁵ Approximately 50% of all choriocarcinomas arise from CHMs, with rare cases of choriocarcinoma arising after a diagnosis of PHM.¹⁶

HMs can be subcategorized into CHMs and PHMs by their unique genetic features. Most CHMs arise from monospermic fertilization of an anucleate ovum, followed by endoreduplication; these cases have a 46,XX karyotype. Less than 10% of CHMs result from dispermic fertilization and can have either a 46,XX or a 46,XY karyotype. In either event, both types of CHM are entirely paternally derived and lack a maternal chromosomal component. However, there are rare familial cases of CHMs that are biparental in origin and contain both maternal and paternal chromosomal components. PHMs arise from either dispermic fertilization of a haploid ovum or monospermic fertilization of a haploid ovum, followed by endoreduplication, and can have the following karyotypes: 69,XXX; 69,XXY; and 69,YYY. Thus, PHMs are, by definition, triploid diandric monogynic molar pregnancies and must be distinguished from triploid monoandric digynic products of conception (POCs), which are nonmolar and, thus, have a different prognosis.¹⁷

The histopathological features of CHMs and PHMs are well described; in many cases, the diagnosis can be made by morphological assessment alone if the classic features are present. However, because of the earlier clinical detection and surgical evacuation of abnormal pregnancies, the histopathological features that are often used to distinguish CHMs, PHMs, and nonmolar abortions (NMAs) are more subtle and less readily identifiable, leading to increasing difficulties in the proper subclassification of HMs.¹⁷ Previ-

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ous studies^{18–20} have shown that there is significant inter-observer variability in the diagnosis of HMs among pathologists.

Because POCs contain both maternal and villous tissue, the genotypes of mother and zygote based on genetic polymorphisms can be used to discern maternal and paternal chromosomal contribution and, thus, aid in the diagnosis of HMs. Polymorphic deletion probes (PDPs) are a recently developed type of fluorescence *in situ* hybridization (FISH) probe that target deletion variants that are highly polymorphic and allow *in situ* determination of genetic identity.²¹ Because PDPs target biallelic polymorphisms, any given individual may have a homozygous FISH genotype (+/+ if both chromosomal loci do not possess the deletion polymorphism or –/– if both loci are deleted) or a heterozygous FISH genotype (+/– if one of two loci does not possess the deletion). PDPs can genetically distinguish between mother and zygote *in situ* and can show an absence of maternal DNA when mutually exclusive genotype pairings are observed: +/+ FISH genotype in decidua and –/– in villi or –/– in decidua and +/+ in villi. Thus, we used a panel of PDPs that target three autosomal deletion loci, one each on chromosomes 2p, 4q, and 8p, to determine the genetic identities of maternal and villous tissue in molar and nonmolar POCs *in situ*. The genotypes of mother and zygote, based on polymorphic deletions, were compared; and ploidy of villous stromal cells was determined to investigate the utility of PDP FISH in the diagnosis of HMs.

Materials and Methods

Tissue Samples

Forty archival cases of POCs, including 13 CHMs, 13 PHMs, 13 NMAs, and an equivocal case of hydropic abortion (HA), diagnosed between January 1, 2004, and December 31, 2009, were retrieved from the obstetric pathology service files of Massachusetts General Hospital, Boston, by searching the pathology electronic database. Clinicopathologic features, including patient age, obstetric history, and clinical impression, were obtained from medical record review. Cytogenetic analysis reports were available for review in two CHMs, six PHMs, four NMAs, and the equivocal HA. Flow cytometry reports were available for review in seven CHMs, eight PHMs, and the equivocal HA. Approval from the Partners Human Research Committee Institutional Review Board was obtained before the initiation of this study.

Histopathological Characteristics

All available H&E-stained slides were reviewed by two pathologists (S.C. and D.J.R.), and the diagnoses were confirmed by consensus. The morphological criteria for the diagnosis of a CHM include a compilation of several of the following features: uniform population of large, round, hydropic villi with budding architecture; prominent cistern formation; moderate to marked, circumferential, trophoblastic proliferation of at least two lineages; stromal karyorrhectic debris; and lack of fetal red blood cells or fetal tissue. A

definitive diagnosis of CHM was made in cases that fulfill the previously mentioned morphological criteria and lack p57 expression by immunohistochemistry (IHC). The diagnosis of a PHM was based on a compilation of several of the following features: a mixed population of normal and moderately hydropic scalloped villi, trophoblastic pseudoinclusions, mild and focal syncytiotrophoblastic proliferation, and the presence of embryonic tissue.¹⁷ A definitive diagnosis of PHM was made in cases that fulfill the morphological criteria for PHM and have confirmation of triploidy by either karyotype or flow cytometry.

IHC Analysis

IHC studies for p57 (p57KIP2 Ab-3 clone KP39 mouse monoclonal antibody; Lab Vision Corporation, Fremont, CA) were performed on all 13 cases of CHM using the avidin-biotin immunoperoxidase method. Heat-induced antigen retrieval was performed on deparaffinized 5- μ m tissue sections at 95°C in 10 mmol/L citrate buffer (pH 6.0) for 30 minutes. Slides were counterstained with hematoxylin. The presence or absence of nuclear staining was evaluated in villous stromal cells, cytotrophoblasts, syncytiotrophoblasts, extravillous trophoblasts, and decidua. Nuclear staining in decidua served as an internal positive control. The p57 immunostain was interpreted as a “positive” result when staining was diffusely positive in all of these cell types. The p57 immunostain was interpreted as a “negative” result if there was complete or near complete (<10% of cells) absence of nuclear staining in villous stromal cells and cytotrophoblasts.

FISH Analysis

Two-color FISH was performed as previously described.²¹ Fosmid clones G248P87627D2 (chromosome 2p PDP) and G248P800808F11 (chromosome 4q PDP) and bacterial artificial chromosome clones RP11-97D17 (chromosome 8p PDP), RP11-460N15 (chromosome 2p control probe), RP11-58C6 (chromosome 4q control probe), and RP11-100B16 (chromosome 8p control probe) were obtained from BAC/PAC Resources (Children’s Hospital Oakland Research Institute, Oakland, CA). Fosmid and bacterial artificial chromosome DNAs were isolated from bacteria with the Qiagen Plasmid Maxi Kit (Qiagen, Valencia, CA), amplified using the REPLI-G Kit (Qiagen), and labeled using a commercial Nick Translation Kit (Abbott Molecular, Abbott Park, IL) with Spectrum Orange-11-dUTP or Spectrum Green-11-dUTP. Briefly, 5- μ m tissue sections from formalin-fixed, paraffin-embedded (FFPE) tissue blocks were mounted on charged slides. A serial H&E-stained section was used to identify well-preserved areas of maternal and villous tissue. After deparaffinization, the unstained sections were subjected to two 30-minute rounds of pepsin digestion at 37°C, followed by wash in 2 \times standard sodium citrate. Slides were air dried, and hybridization mix (3 μ L/slide) containing the appropriate PDP (labeled orange) and a nonpolymorphic control probe (labeled green) was applied to the slides, followed by denaturation of the probe and target at 80°C for 5 minutes and overnight hybridization at 37°C. Two 5-minute posthybridization washes in 2 \times standard sodium

citrate were performed at 37°C. Nuclei were counterstained with DAPI. Images were acquired with an Olympus BX61 fluorescent microscope equipped with a charge-coupled device camera and analyzed with Cytovision software (Genetix, San Jose, CA).

The genotypes of mother and zygote, as detected by PDP FISH, were recorded and agreed on by two pathologists (S.C. and A.J.I.). Only maternal decidual or tubal stromal cells and villous stromal cells with at least two copies of the nonpolymorphic control probe were scored. For diploid cells, the following genotypes were possible: homozygous (+/+ and -/-) and heterozygous (+/-). For triploid cells, the following genotypes were possible: homozygous (+/+/+ and -/-/-) and heterozygous (+/+/- and +/-/-). 8p PDP control probe signal quantitation of 50 villous stromal cell nuclei (because of multinucleation within syncytiotrophoblasts, only villous stromal cells were scored) in each case of NMA, CHM, and PHM were used to generate a signal threshold that would allow for determination of triploidy.

FISH Immunofluorescence

Combined 8p PDP FISH and immunofluorescence was performed on deparaffinized 5- μ m tissue sections using a modified avidin-biotin immunofluorescence method. Pressure-cooker antigen retrieval was performed by heating tissue sections in Borg Decloaker solution (Biocare Medical, Concord, CA) for 3 minutes, followed by cooling sections to room temperature. Slides were washed and incubated in PBS buffer for 5 to 10 minutes. Slides were then incubated in avidin D for 20 minutes, biotin for 20 minutes, mouse anti-p57 (1:200 dilution in PBS) for 60 minutes, horse anti-mouse IgG (1:100 dilution in PBS) (Vector Labs, Burlingame, CA) for 30 minutes, and Cy5-streptavidin (1:100 dilution in PBS) (Invitrogen, Camarillo, CA) for 30 minutes between washes in PBS. Slides were dehydrated in ethanol and dried in a 65°C oven for 5 minutes. Two-color FISH using chromosome 8p PDP was performed as previously described. Posthybridization washes were performed in 0.4 \times standard sodium citrate/0.3% NP-40 at 72°C for 2 minutes, followed by 2 \times standard sodium citrate/0.1% NP-40 at room temperature for 1 minute.

Statistical Analysis

A definitive diagnosis of CHM can be made when the maternal and villous genotypes are mutually exclusive in at least one of the three PDP loci. In cases without mutually exclusive maternal and villous genotypes, the likelihood of the observed villous genotype and the maternal genotype was calculated under two possible cases: CHM and NMA. For the likelihood computation under the assumption of CHM, there were two subclasses to consider: dispermic fertilization and haploid fertilization. Based on a literature search, the frequency of dispermic fertilization was assumed to be 3% to 29%. A likelihood ratio was then computed to compare the likelihood of the observed data under the assumption of CHM relative to the likelihood of the observed data under the assumption of NMA. A large ratio indicates that the data are more supportive of CHM than

NMA. This served to order the possible outcomes and thereby provide a rationale diagnostic algorithm specifically for the cases lacking mutually exclusive genotypes. Although the actual likelihood ratio is a function of the unknown frequency, the ordering of observed outcomes is independent of this unknown frequency. The sensitivity and specificity of the sequence of resulting diagnostic algorithms were then computed.

Based on theoretical calculations, the appropriate nested sequence of diagnostic algorithms for CHM for consideration is as follows:

1. Diagnose CHM when maternal and villous FISH genotypes are mutually exclusive. This has theoretical 100% specificity and 56% sensitivity (assuming that the rate of dispermic fertilization is 10%).
2. Diagnose CHM when maternal and villous genotypes are mutually exclusive or when the villous tissue is homozygous at all three probes, regardless of the maternal genotype. This has theoretical 87.5% specificity and 91.8% sensitivity.

For the purpose of diagnosing PHMs, the frequencies of triploid cells were estimated using the 13 cases of PHM, the 13 cases of CHM, and the 13 NMAs and their 8p PDP and control results. The rates were estimated using those cells for which there were one, two, or three control probes. The estimates of triploidy were 0.8% among the NMAs, 1.2% to 3.4% among the CHMs, and 45.3% among the PHMs. Given these widely disparate estimates, it was assumed that the true rate for NMAs and CHMs is <10% and that the true rate for PHMs is >35%. Based on this assumption, a cutoff for diagnosing PHM was derived, with high sensitivity and specificity (based on simple binomial probability calculations).

Results

Clinicopathologic Features

Forty FFPE POC specimens with consensus final pathological diagnoses of NMA, PHM, CHM, and an equivocal HA were obtained from the pathology archives of Massachusetts General Hospital. The age of the patients ranged from 24 to 41 years (mean, 33.4 years) for those with NMAs, from 20 to 53 years (mean, 32.7 years) for those with CHMs, and from 29 to 39 years (mean, 33.5 years) for those with PHMs. The age of the patient with the equivocal HA was 21 years. There were no statistically significant differences between the ages of women with CHMs, PHMs, and NMAs. Of 40 patients, 35 (87.5%) had at least one prior pregnancy. Of 13 patients in each group, six (46.2%) with CHMs, five (38.5%) with PHMs, and eight (61.5%) with NMAs had at least one prior abortion; however, none of these patients had a history of molar pregnancy. Molar pregnancy was clinically suspected in 10 (76.9%) of 13 CHMs, nine (69.2%) of 13 PHMs, and the equivocal HA. The remaining cases were considered missed abortions. Of the NMAs, eight cases (61.5%) were missed abortions, two cases (15.4%) were ectopic pregnancies, and three cases (23.1%) were therapeutic abortions. The clinicopathologic features for CHMs,

Table 1. Clinicopathologic Features, IHC, and Molecular Genetic Analysis of CHMs

Case no.	Age (years)	Obstetric history	p57	Karyotype	FC	FISH						Final diagnosis
						2p		4q		8p		
						DE	CV	DE	CV	DE	CV	
1	33	G ₄ P ₁₀₂₁ with mole	N	46,XX	ND	+/+	+/+	-/-*	+/+*	+/-	+/+	CHM
2	34	G ₆ P ₁₀₄₁ with MAB	N	ND	D	+/+*	-/-*	+/-	+/+	+/-	-/-	CHM
3	34	G ₁ P ₀₀₀₀ with CHM	N	ND	D	-/-*	+/+*	+/-	+/+	+/+*	-/-*	CHM
4	34	G ₂ P ₁₀₀₁ with CHM	N	ND	D	+/+*	-/-*	+/+	+/+	+/-	+/+	CHM
5	26	G ₁ P ₀₀₀₀ with CHM	N	ND	ND	+/+	+/+	+/+*	-/-*	+/-	+/+	CHM
6	53	G ₄ P ₃₀₀₃ with mole	N	ND	ND	+/+	+/+	+/-	+/+	+/+	+/+	CHM
7	28	G ₂ P ₁₀₀₁ with MAB	N	ND	D	+/+	+/+	+/+	+/+	+/+	+/+	CHM
8	27	G ₂ P ₁₀₀₁ with CHM	N	46,XX	ND	+/-	+/+	+/+	+/+	+/-	+/+	CHM
9	40	G ₂ P ₀₀₁₀ with CHM	N	ND	D	+/+	+/+	+/+	+/+	+/+	+/+	CHM
10	20	G ₂ P ₀₀₁₀ with CHM	N	ND	D	+/-	+/+	+/-	-/-	+/-	-/-	CHM
11	22	G ₂ P ₀₀₁₀ with MAB	N	ND	ND	+/+	+/+	+/+	+/+	+/+	+/+	CHM
12	35	G ₇ P ₆₀₀₀ with mole	N	ND	D	+/+	+/+	+/-	+/+	+/+	+/+	CHM
13	40	G ₃ P ₁₀₁₁ with mole	N	ND	ND	+/-	+/+	+/-	-/-	-/-	-/-	CHM

*Cases 1 to 5 represent CHM in which DE and CV show informative mutually exclusive FISH results.

CHM, complete hydatidiform mole; CV, chorionic villi; D, diploid; DE, decidua; FC, flow cytometry; G, gravida; MAB, missed abortion; N, negative; ND, not done; P, para (term, preterm, abortion, living).

NMAs, PHMs, and the equivocal HA are summarized in Table 1, Table 2, Table 3, and Table 4, respectively.

Karyotyping data were available for four NMAs, two CHMs, six PHMs, and the equivocal HA. Three NMAs showed the following abnormal karyotypes: 45,X; 47,XY,+13; and 47,XY,+13,rob(13;13)(q10;q10)dn. Both CHMs were diploid and homozygous (46,XX). All six PHMs showed triploidy (two cases of 69,XXX; three cases of 69,XXY; and one case of 70,XXY,+8). Cytogenetic analysis of the equivocal HA was limited by poor cell growth and showed 10 metaphases, six of which were diploid with a female complement, three XX missing one or two autosomes, and one XXXX with >90 chromosomes. Flow cytometry data were available for seven CHMs, eight PHMs, and the equivocal HA. All seven CHMs were diploid. Seven PHMs demonstrated triploidy, and one case showed mixed diploidy and triploidy. The equivocal HA was diploid. p57 immunostaining was performed on all 13 cases of CHM and

demonstrated complete or near-complete loss of p57 expression in the villous stromal cells and cytotrophoblasts, confirming paternal chromosomal contribution and the diagnosis of CHM. Appropriate positive immunoreactivity in the decidua was present. Antibody-negative controls were negative (data not shown).

PDP FISH Analysis

FISH analysis was performed on paraffin sections of all 40 POC specimens using 2p, 4q, and 8p PDPs to assess for the genetic identity of maternal (decidua) and zygotic (villous) tissues. Nonpolymorphic FISH probes adjacent to the 2p, 4q, and 8p loci were simultaneously examined to control for sectioning artifacts and allow for ploidy determination.

We first examined the villous tissue in the 13 CHMs and found all to be diploid and homozygous for all

Table 2. Clinicopathologic Features and Molecular Genetic Analysis of NMAs

Case no.	Age (years)	Obstetric history	Karyotype	FISH						Final diagnosis
				2p		4q		8p		
				DE	CV	DE	CV	DE	CV	
14	31	G ₂ P ₁₀₀₁ with MAB	45,X	+/+	+/+*	+/+	+/+*	+/+	+/+*	NMA
15	30	G ₂ P ₁₀₀₁ with TAB	ND	+/+	+/+	+/+	+/+	-/-	+/-	NMA
16	32	G ₄ P ₁₀₂₁ with EP	ND	+/-	+/+	+/+	+/-	+/+	+/-	NMA
17	34	G ₂ P ₁₀₀₁ with MAB	ND	+/+	+/+	+/-	-/-	+/-	+/-	NMA
18	29	G ₂ P ₀₀₁₀ with EP	ND	+/-	+/-	+/-	-/-	+/+	+/-	NMA
19	35	G ₄ P ₀₀₃₀ with MAB	46,XY	-/-	-/-	+/-	+/-	+/-	+/+	NMA
20	41	G ₃ P ₀₀₂₀ with MAB	47,XY,+13	+/+	+/-	+/+	+/+	+/+	+/-	NMA
21	35	G ₁ P ₀₀₀₀ with MAB	ND	+/+	+/+	+/+	+/-	+/-	-/-	NMA
22	33	G ₅ P ₂₀₂₂ with TAB	ND	-/-	-/-	+/+	+/+	+/-	+/-	NMA
23	24	G ₇ P ₂₀₄₂ with TAB	ND	-/-	-/-	+/+	+/-	-/-	+/-	NMA
24	38	G ₂ P ₀₀₁₀ with MAB	ND	-/-	-/-	+/+	+/+	+/-	+/-	NMA
25	41	G ₅ P ₂₀₂₂ with MAB	ND	+/-	+/-	+/-	-/-	+/-	+/+	NMA
26	31	G ₂ P ₁₀₀₁ with MAB	47,XY,+13,rob(13;13)(q10;q10)dn	+/+	+/+	+/-	+/+	+/-	+/-	NMA

*Case 14 demonstrates homozygosity in villous stromal cells with all three probes.

CV, chorionic villi; DE, decidua; EP, ectopic pregnancy; G, gravida; MAB, missed abortion; ND, not done; NMA, nonmolar abortion; P, para (term, preterm, abortion, living); TAB, therapeutic abortion.

Table 3. Clinicopathologic Features and Molecular Genetic Analysis of PHMs

Case no.	Age (years)	Obstetric history	Karyotype	FC	FISH						Final diagnosis
					2p		4q		8p		
					DE	CV	DE	CV	DE	CV	
27	31	G ₅ P ₁₁₂₃ with PHM	69,XXY	ND	+/-	+/+/-	+/+	+/+/-	-/-*	+/+/-*	PHM
28	34	G ₁ P ₀₀₀₀ with mole	ND	T	+/-	-/-/-	-/-*	+/+/-*	+/-	+/-/-	PHM
29	31	G ₄ P ₂₀₁₃ with MAB	ND	D/T	+/-	+/+/-	+/+*	+/+/-*	+/-	+/-/-	PHM
30	29	G ₂ P ₁₀₀₁ with mole	ND	T	+/+*	+/+/-*	+/+	+/+/-	+/-	+/+/+	PHM
31	37	G ₂ P ₁₀₀₁ with PHM	69,XXY	ND	+/+	+/+/+	-/-*	+/+/-*	+/-	+/+/+	PHM
32	39	G ₂ P ₁₀₀₁ with PHM	70,XXY,+8	ND	+/-	+/+/-	+/-	+/+/-	-/-*	+/+/+/-*	PHM
33	31	G ₃ P ₀₀₀₀ with MAB	ND	T	+/-	+/+/+	+/-	+/+/+	+/-	+/+/-	PHM
34	34	G ₂ P ₁₀₀₁ with MAB	ND	T	+/-	+/+/-	+/+	+/+/+	-/-	-/-/-	PHM
35	34	G ₆ P ₃₀₂₃ with MAB	ND	T	+/-	+/+/+	+/+	+/+/+	+/-	+/+/-	PHM
36	29	G ₂ P ₁₀₀₀ with mole	69,XXX	T	+/+	+/+/+	+/-	+/+/+	-/-	-/-/-	PHM
37	37	G ₂ P ₀₀₁₀ with PHM	ND	T	-/-	-/-/-	+/-	-/-/-	+/-	+/-/-	PHM
38	31	G ₂ P ₀₁₀₁ with PHM	69,XXX	ND	+/-	+/+/-	+/-	+/+/-	+/-	+/+/-	PHM
39	39	G ₆ P ₂₀₃₂ with PHM	69,XXY	ND	+/-	+/+/-	+/-	+/+/-	+/-	+/-/-	PHM

*Cases 27 to 32 represent definitive diandric monogynic triploidy.

CV, chorionic villi; D, diploid; DE, decidua; FC, flow cytometry; G, gravida; MAB, missed abortion; ND, not done; P, para (term, preterm, abortion, living); PHM, partial hydatidiform mole; T, triploid.

three PDPs (Table 1). PDP FISH allowed for a definitive diagnosis of CHM in five cases (38.5%) in which the maternal and villous genotypes were mutually exclusive for at least one PDP locus (mutually exclusive genotypes are either +/+ in decidua and -/- in villi or -/- in decidua and +/+ in villi), thus indicating a paternal-only genetic contribution (Figure 1). If we were to take mutual exclusivity as our diagnostic rule (rule 1), then we estimate that we have 56% sensitivity and 100% specificity (if the frequency of dispermic fertilization is 10%). Our observed sensitivity is 38.5% (5/13), and our actual specificity (based on Table 2 for NMAs) is 100%. In the remaining eight cases, we could not prove paternal-only contribution because the maternal tissue was heterozygous or shared the same homozygous genotype as the villous tissue. Because only 38.5% of CHMs could be definitively diagnosed using the mutual exclusion criteria, we considered application of our likelihood ratio-based diagnostic algorithms derived from PDP FISH data on NMAs (Figure 2 and Table 2). If we diagnose CHM in cases that demonstrate homozygosity in villous tissue for all three PDPs regardless of maternal genotype, then we estimate that we have approximately 91.8% sensitivity and

87.5% specificity (again, if the frequency of dispermic fertilization is 10%). Based on our experimental PDP data in Table 1, we would diagnose all 13 cases (cases 1 to 13) as CHM because they were homozygous for all three probes, increasing our empirical sensitivity to 100%, with an observed specificity of 92.3% because the 13 cases of NMA showed only one case (7.7%) with homozygosity for all three PDPs.

FISH Confirmation of Triploidy in PHMs

PDP FISH demonstrated triploidy in villous tissue for all nonpolymorphic 8p control probes in all 13 PHM cases (Figure 3 and Table 3). Based on our estimates of rates of triploidy of <10% for NMAs and CHMs and >35% for PHMs, exact binomial probability calculations yield the simple rule that if FISH copy number in 50 villous stromal nuclei with one, two, or three nonpolymorphic control probe signals is determined, a diagnosis of triploidy can be declared if ≥ 11 ($\geq 22\%$) nuclei have a copy number of three (sensitivity, 98.4%; specificity, 98.1%). If <11 (<22%) nuclei have a copy number of three, a diagnosis of diploidy can be declared.

Table 4. Clinicopathologic Features and Molecular Genetic Analysis of a Possible Twin Gestation with a CHM and a Fetus

Case no.	Age (years)	Obstetric history	Karyotype	FC	FISH												Final diagnosis
					2p			4q			8p						
					CV			CV			CV						
					DE	VC	VS	FP	DE	VC	VS	FP	DE	VC	VS	FP	
40	21	G ₁ P ₀₀₀₀ with PHM	6 XX, 1 XXXX, 3 XX missing autosomes	D	+/-	+/+	+/+	+/+	+/-	-/-	-/-	-/-	+/-	+/-	+/+	+/+	Singleton pregnancy with a mosaic androgenetic/biparental placenta

CV, chorionic villi; D, diploid; DE, decidua; FC, flow cytometry; FP, fetal parts; G, gravida; P, para (term, preterm, abortion, living); PHM, partial hydatidiform mole; VC, villous cytotrophoblast; VS, villous stroma.

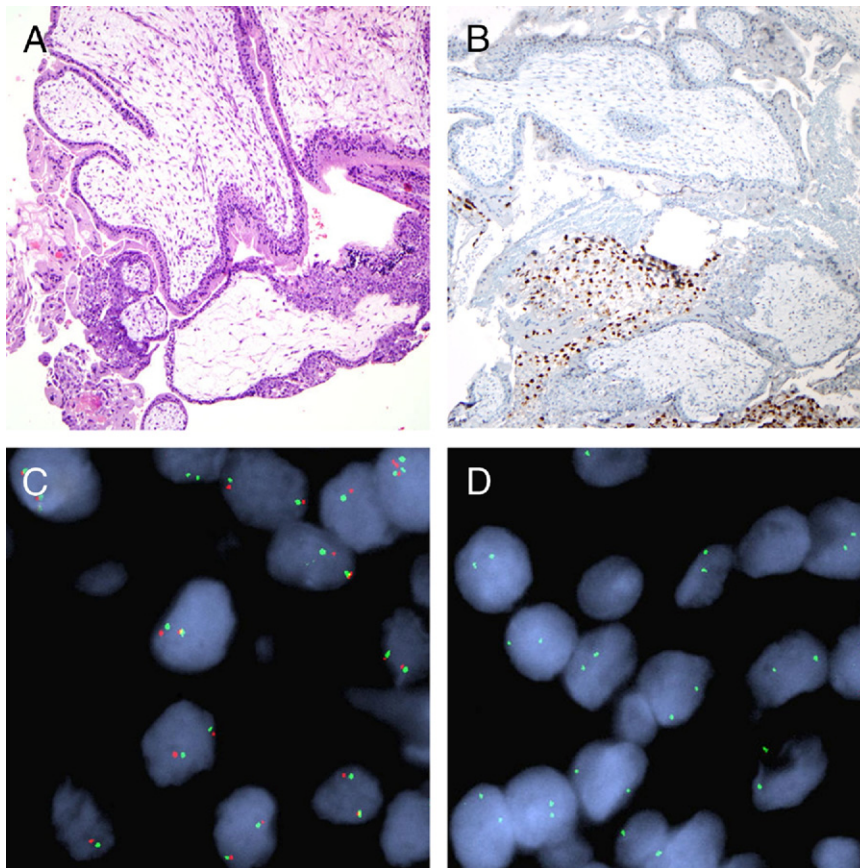


Figure 1. CHM with informative PDP FISH results. **A:** CHM is characterized by large hydropic villi with budding architecture, circumferential trophoblastic hyperplasia, and cellular stroma containing karyorrhectic debris (H&E staining). Original magnification, $\times 100$. **B:** IHC studies show lack of p57 expression in villous stroma, cytotrophoblasts, and syncytiotrophoblasts and persistent p57 expression in extravillous cytotrophoblasts. Original magnification, $\times 100$. **C** and **D:** Chromosome 2p PDP FISH analysis performed on the villous stroma and decidua reveals a homozygous genotype (+/+) in villous stroma (**C**) and a homozygous genotype (-/-) in decidua (**D**). Red indicates chromosome 2p PDP; and green, control nonpolymorphic probe.

An examination of PDP probes revealed six cases (46.2%) with definitive diandric monogynic triploidy based on the following genotype pairings: +/+ in decidua and +/-/- in villi or -/- in decidua and +/+/- in villi. Two cases demonstrated +/+ genotypes in decidua and +/+/- genotypes in villi, suggesting either a digynic triploid gestation, which is nonmolar, or a PHM, which may have resulted from dispermic fertilization by a sperm with a + genotype and a sperm with a - genotype. Digynic triploidy could not be excluded in seven cases.

Androgenetic/Biparental Mosaic Placenta Diagnosed by PDP FISH

PDP FISH was used to analyze an unusual HA. A 21-year-old primigravida woman at 17 weeks' gestation presented with absent fetal heart rate and a limited pelvic ultrasonogram that demonstrated a large cystic placenta and a single intrauterine gestational sac containing a fetal pole with no fetal heart motion, clinically consistent with a PHM. Serum human chorionic gonadotropin at 17 weeks' gestation

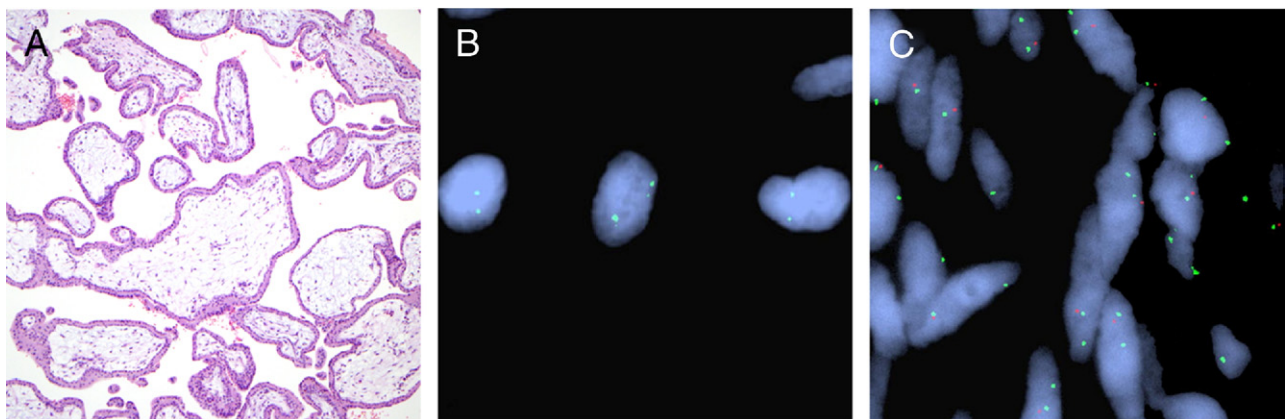


Figure 2. PDP FISH in an NMA. **A:** Routine H&E staining of a tubal ectopic pregnancy demonstrates normal villous morphological features. Original magnification, $\times 100$. **B** and **C:** Chromosome 8p PDP FISH analysis performed on the villous stroma (**B**) and tubal stroma (**C**) reveals a homozygous genotype (-/-) in villous stroma and a heterozygous genotype (+/-) in tubal stroma. Red indicates chromosome 8p PDP; and green, control nonpolymorphic probe.

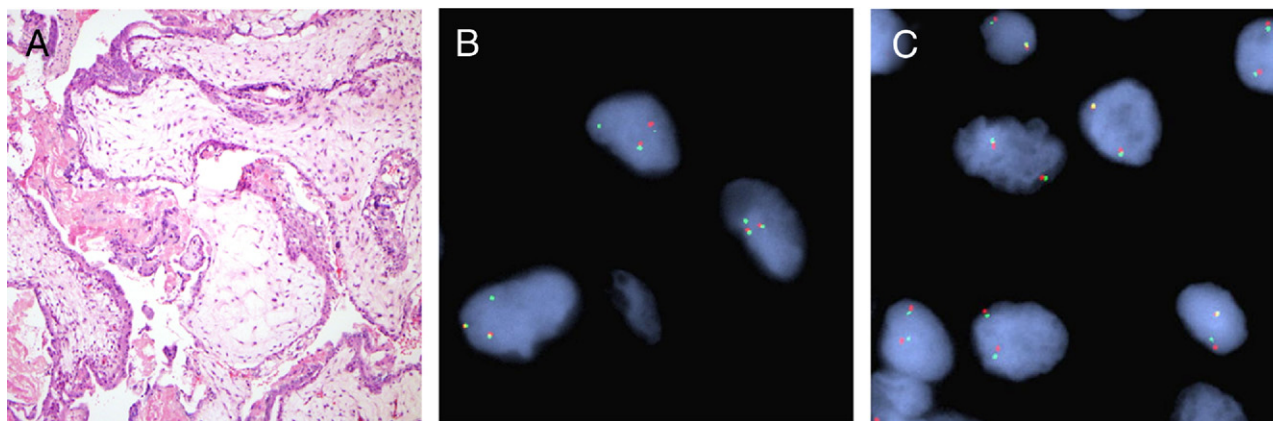


Figure 3. PDP FISH demonstrates triploidy in PHMs. **A:** PHM is characterized by irregular villi with focal mild trophoblastic hyperplasia and trophoblastic pseudoinclusions (H&E staining). Original magnification, $\times 100$. **B** and **C:** Chromosome 4q PDP FISH analysis performed on the villous stroma and decidua reveals a heterozygous genotype (+/+/-) in the villous stroma and a homozygous genotype (+/+) in the decidua. Red indicates chromosome 4q PDP; and green, control nonpolymorphic probe.

was measured at 242,048 mIU/mL (normal range is 4060 to 165,400 mIU/mL for a gestational age of 17 to 24 weeks). Dilation and curettage was performed, and gross examination of the POCs revealed large bulbous villi admixed with fragments of autolyzed fetus with an estimated gestational age of <12 weeks. Only fragments of fetal extremities and skull were present; no organ tissues were identified. Microscopic sections of the villous tissue showed focal trophoblastic proliferation associated with two morphologically distinct populations of chorionic villi: swollen hydropic villi with central cisterns and smaller slightly irregular villi (Figure 4). The initial pathological impression was of a twin gestation with a CHM and an autolyzed fetus of <12 weeks' gestational age.

Three-probe PDP FISH was performed and demonstrated diploidy and homozygous genotypes in the villous stromal cells of both large and small villi for all probes. However, 8p PDP FISH showed heterozygous genotypes in the villous cytotrophoblasts and syncytiotrophoblasts (Figure 4). Combined 8p PDP FISH and p57 immunofluorescence demonstrated p57 expression in the heterozygous cytotrophoblasts and syncytiotrophoblasts and absence in the homozygous villous stromal cells, indicating a genetically mosaic placenta consistent with the diagnosis of androgenetic/biparental mosaicism (Figure 4). PDP FISH of the fetal bone marrow demonstrated homozygous genotypes with the 2p and 4q probes and a heterozygous genotype with the 8p probe, identical to the genotype of the villous cytotrophoblasts and syncytiotrophoblasts and consistent with a fetus resulting from a biparental conception. Based on the pathological and molecular genetic features of this case, the patient was observed clinically by weekly serum human chorionic gonadotropin level, like any patient with a CHM. Her last six measurements of serum human chorionic gonadotropin were <6 mIU/mL over 4 months. There has been no recurrence of disease 7 months since her initial presentation. The clinicopathologic features and molecular genetic analysis results are summarized in Table 4.

Discussion

We performed molecular genetic analysis of HMs using PDP FISH to assess the usefulness of this novel technique in diagnosing and distinguishing different subtypes of molar pregnancies. With a panel of three PDPs, FISH can definitively diagnose approximately 40% of CHMs based on mutually exclusive genotypes of mother and zygote. This is based on the small sample size of 13; theoretically, this sensitivity is 56%. For cases in which CHM is suspected, but PDP FISH results are not mutually exclusive, our technique supports a diagnosis of CHM in cases in which the zygotic genotype is homozygous for all three probes, regardless of the maternal genotype (sensitivity, 91.8%; specificity, 87.5%). PDP FISH (using control probes for copy number determination) can also serve as ploidy analysis and confirm triploidy in PHMs (sensitivity, 98.4%; specificity, 98.1%). Furthermore, it can distinguish triploid diandric PHMs from triploid digynic nonmolar specimens by readily identifying the parental source of chromosomal contribution in approximately 50% of cases in which a PHM is suspected.

Several ancillary studies have been used clinically to improve the diagnosis of HMs, including IHC staining for the paternally imprinted *p57* gene, cytogenetic analysis, and ploidy analysis by flow cytometry or FISH. Because most CHMs lack maternal genomic content and are not expected to express the paternally imprinted *p57* gene, *p57* immunostaining has become a valuable tool in the diagnosis of CHMs and can be easily performed on paraffin-embedded tissue.^{22–24} However, interpretation of the *p57* immunostains can be difficult; and *p57* immunostaining alone cannot distinguish PHMs from NMAs because both entities contain a maternal chromosomal component and retain *p57* expression. Thus, recent studies^{25–28} have combined *p57* IHC with a molecular technique to aid in the diagnosis of HMs. Karyotyping can identify chromosomal numerical and structural abnormalities that can help in the diagnosis of HMs; however, this technique requires fresh tissue, is labor and time inten-

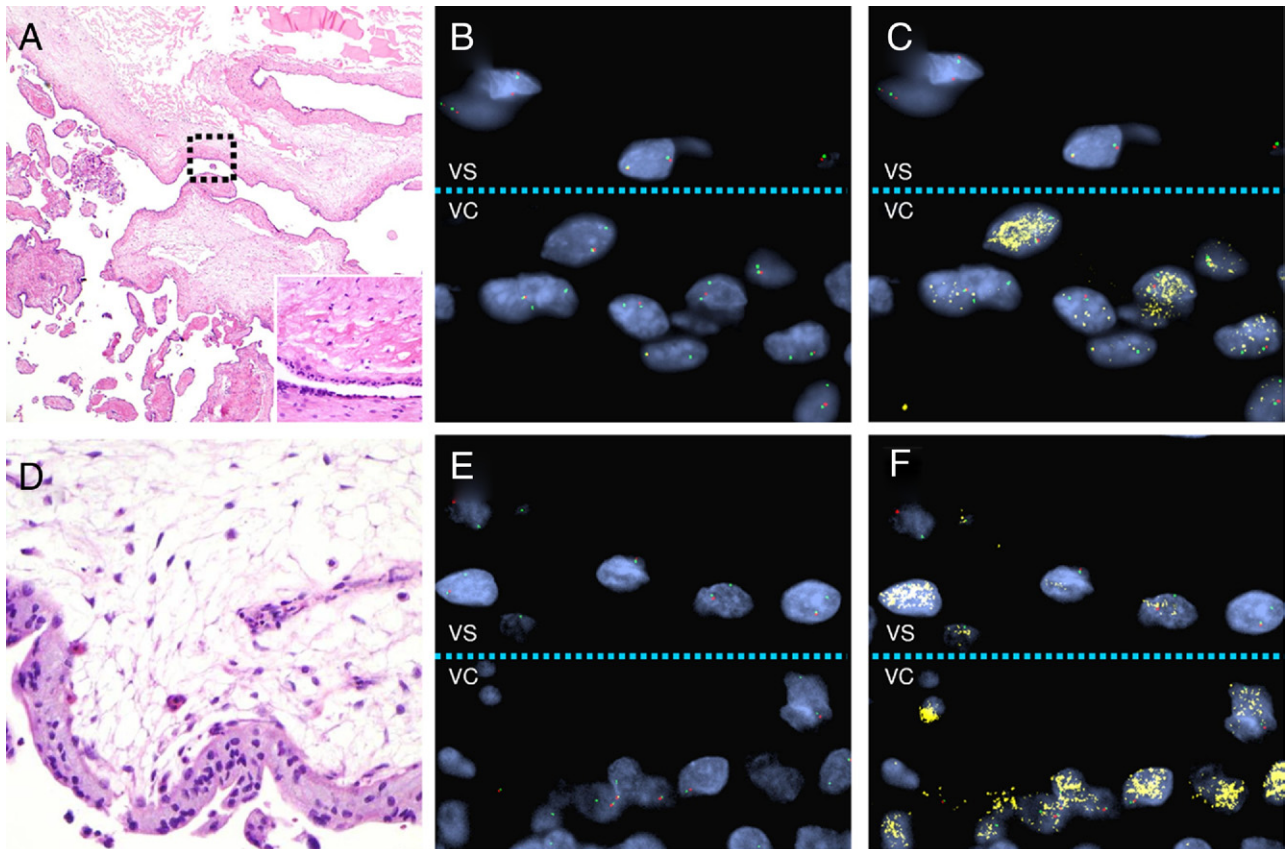


Figure 4. A singleton pregnancy consisting of a fetus with a mosaic androgenetic/biparental placenta, confirmed by combined 8p PDP FISH and p57 immunofluorescence (IF). **A:** Sections demonstrate a mixture of large hydropic villi with central cistern formation (**inset**: $\times 400$), smaller irregular villi, and focal trophoblastic proliferation (H&E staining). Original magnification, $\times 40$. **B:** Chromosome 8p PDP FISH analysis of the tissue in **A** reveals a homozygous genotype (+/+) in the villous stroma (VS) (nuclei above the teal dotted line) and a heterozygous genotype (+/-) in the villous cytotrophoblasts (VCs) (nuclei below the teal dotted line). **C:** p57 IF superimposed on the same section in **B** demonstrates diffuse p57 expression within the nuclei of VCs that have a heterozygous genotype (+/-) and absence of p57 expression within the nuclei of VS cells that have a homozygous genotype (+/+). **D:** First-trimester villi in an NMA show rare small blood vessels containing fetal nucleated red blood cells and no trophoblastic hyperplasia (H&E staining). Original magnification, $\times 400$. **E:** Chromosome 8p PDP FISH analysis of the tissue in **D** reveals a heterozygous genotype (+/-) in the VS and the VCs. **F:** p57 IF superimposed on the same section in **E** demonstrates diffuse p57 expression within all nuclei in VS cells and VCs.

sive, and is not readily available in all cases.²⁹ Ploidy analysis by flow cytometry or FISH can be performed on fresh or paraffin-embedded tissue and can differentiate between diploid and triploid POCs.^{30–32} However, both

karyotyping and ploidy analysis are subject to maternal contamination; neither technique can specifically discern maternal from paternal chromosomal contribution, which is essential in the diagnosis of molar pregnancies.

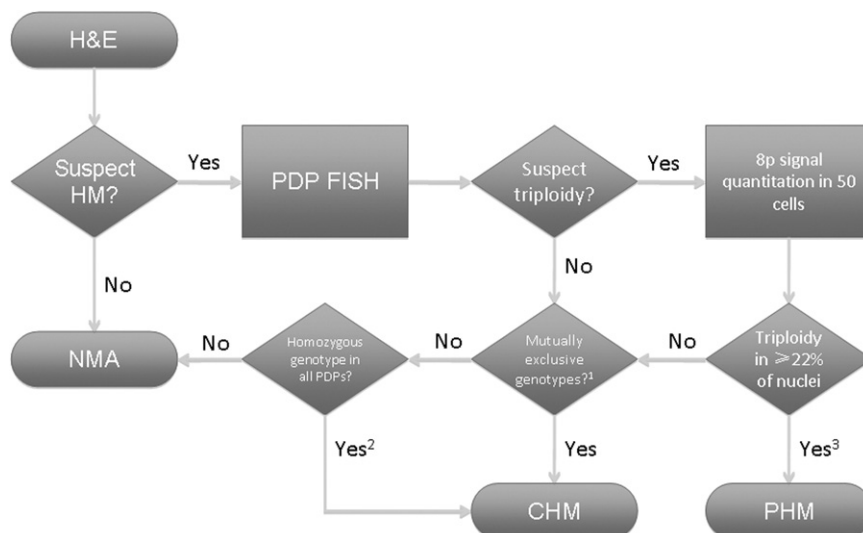


Figure 5. Algorithm for PDP FISH analysis of a suspected molar pregnancy. ¹Mutually exclusive genotype pairings (+/+ in decidua and -/- in villi or -/- in decidua and +/+ in villi) at any PDP locus provide a definitive diagnosis of CHM. ²If villous genotypes are homozygous for all PDPs regardless of the maternal genotype, the diagnosis is likely a CHM (sensitivity, 91.8%; specificity, 87.5%). ³A definitive diagnosis of PHM is made if, for any PDP locus, the zygotic genotype is +/-/- and the maternal genotype is -/- or the zygotic genotype is +/-/- and the maternal genotype is +/+. If these criteria are not met, but triploidy is confirmed if $\geq 22\%$ of nuclei each demonstrate three nonpolymorphic probe signals by 8p signal quantitation in 50 villous stromal nuclei, the diagnosis is likely a PHM, but digynic triploidy cannot be excluded.

PCR-based genomic fingerprinting with short tandem repeat (STR) polymorphisms, which is the current gold standard molecular genetic technique used in identity testing, is gaining use in confirming paternal origin of HMs in investigational studies. STRs are repeated sequences of two or more nucleotides typically located in noncoding intron regions of the human genome. They are highly polymorphic and can serve as robust markers of genetic identity. By identifying the genotypes of maternal and villous tissues at multiple STR loci and determining the parental source of polymorphic alleles and their ratios in villous tissues, STR assays can classify molar pregnancies. Several recent studies^{26,27,31,33–35} have shown that STR genotyping can accurately distinguish between androgenetic diploid CHMs and biparental diploid NMAs and between triploid diandric monogynic PHMs and triploid digynic monoandric nonmolar POCs. STR genotyping can be performed on paraffin-embedded tissue. Because it is a PCR-based technique, STR analysis may be limited by poor amplification of DNA because of limited tissue sampling; however, many of the commercially available multiplex STR assays have been optimized for even small quantities of DNA. STR genotyping also requires pure populations of maternal and villous tissues; maternal contamination can confound the interpretation of genotype data, resulting in misclassification. *In situ* analysis avoids the technically challenging requirement of purifying maternal and villous tissues before analysis.

PDP FISH is a novel molecular genetic technique that utilizes deletion copy number variants to determine genetic identity. As shown in the current study, it can discern maternal and paternal chromosomal contribution in CHMs, NMAs, and a large subset of PHMs. Deletion variants are a subclass of copy number variants that are highly polymorphic and can potentially serve as markers of genetic identity.^{36–38} The major value of PDP FISH lies in its ability to assess genetic identity *in situ*, especially in cases of small sample size.²¹ Genotyping and concomitant assessment of cell and tissue morphological features in PDP FISH offer a competitive advantage over other methods used in clinical and investigational studies. Furthermore, PDP FISH can be easily performed on FFPE tissue. Although both maternal and villous tissues are necessary to determine paternal contribution, genotypes can be accurately identified, even with small tissue samples. When coupled with other ancillary techniques, such as p57 immunofluorescence, PDP FISH may be a useful diagnostic tool in the evaluation of mosaicism and chimerism that can occur with HAs. We have developed a diagnostic algorithm for the interpretation of PDP FISH in the evaluation of POCs in which the diagnosis of HM is suspected (Figure 5). PDP FISH may also be used to study both normal and abnormal placentation.

A few technical limitations exist for *in situ* copy number assays in the genetic analysis of HMs. Sectioning artifact can lead to the misinterpretation of genotype data. This can be minimized by using a nonpolymorphic copy number control probe that is located near the PDP locus (<1.0 megabase) and analyzing nuclei that have at least two copies of the nonpolymorphic copy number control probe. FISH signals in syncytiotrophoblasts can be difficult to interpret because of multinucleation and overlapping nuclei.

We limited our analysis of villous tissue to cytotrophoblasts and villous stromal cells that are morphologically distinct and easily identified with a fluorescence microscope. Although our panel of three PDPs can definitively diagnose diandric triploidy in only a proportion of PHMs, we anticipate that the development of additional probes that target other potential deletion polymorphisms can substantially increase the discriminative ability of a multiprobe approach in the analysis of HMs (with two additional probes that can definitively diagnose CHM with 75% sensitivity). We are developing FISH probes for other deletion loci. Sole use of PDP FISH may not be able to definitively diagnose rare dispermic CHMs, biparental CHMs, or CHMs with retained maternal copies of chromosomes 2, 4, and 8. As with other ancillary studies used in the evaluation of HMs, PDP FISH cannot be used alone and must be combined with prior assessment of villous morphological features. If a molar gestation is suspected based on the morphological impression and PDP FISH is not definitive, further investigation with other molecular techniques, such as STR genotyping, may be useful. Although our current study demonstrates a successful application of PDP FISH in a small cohort of molar gestations, future independent studies involving a larger sample size are necessary should PDP FISH be used in clinical practice.

In summary, PDP FISH is an accurate and practical molecular ancillary technique for the classification of HMs and may provide insights into the study of unusual POCs, including mosaic and chimeric conceptions.

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